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## Reduced Lysis upon Growth of *Lactococcus lactis* on Galactose Is a Consequence of Decreased Binding of the Autolysin AcmA<sup>∇</sup>

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When *Lactococcus lactis* subsp. *lactis* IL1403 or *L. lactis* subsp. *cremoris* MG1363 is grown in a medium with galactose as the carbon source, the culture lyses to a lesser extent in stationary phase than when the bacteria are grown in a medium containing glucose. Expression of AcmA, the major autolysin of *L. lactis*, is not influenced by the carbon source. Binding studies with a fusion protein consisting of the MSA2 protein of *Plasmodium falciparum* and the C-terminal peptidoglycan-binding domain of AcmA revealed that cell walls of cells from both subspecies grown on galactose bind less AcmA than cell walls of cells grown on glucose. Cells grown on glucose or galactose and treated with trichloroacetic acid prior to AcmA binding bind similar amounts of AcmA. Analysis of the composition of the lipoteichoic acids (LTAs) of *L. lactis* IL1403 cells grown on glucose or galactose showed that the LTA composition is influenced by the carbon source: cells grown on galactose contain LTA with less galactose than cells grown on glucose. In conclusion, growth of *L. lactis* on galactose changes the LTA composition in the cell wall in such a way that less AcmA is able to bind to the peptidoglycan, resulting in a decrease in autolysis.

Gram-positive bacteria produce enzymes that hydrolyze peptidoglycan (PG), the major component of the cell wall (14). The model organism for lactic acid bacteria, *Lactococcus lactis*, produces three types of PG hydrolases: four *N*-acetyl-glucosaminidases (AcmA, AcmB, AcnC, and AcmD); two putative gamma-D-glutamyl-L-lysyl-endopeptidases (YjgB and lmg\_0506), both containing an NlpC/P60 domain (Pfam PF00877); and four putative amidases (AcmB, Usp45, lmg\_0904, and lmg\_1890) with a CHAP (cysteine, histidine-dependent amidohydrolases/peptidases) domain (Pfam PF05257) (37). AcmB contains the glucosaminidase active-site domain in the N terminus of the mature protein and the CHAP domain in its C terminus. AcmA is responsible for stationary-phase lysis (autolysis) and bacteriocin-induced lysis of *L. lactis* subsp. *cremoris* MG1363 and is involved in cell separation of this bacterium (8, 30). AcmB is not involved in cell separation but does contribute to autolysis (20). The function of AcnC and AcmD has not been elucidated, but increased expression of AcnC has been shown to result in increased cellular lysis independent of the presence of AcmA (21, 45). The genes for these PG hydrolases are present in the chromosomes of the three sequenced *L. lactis* strains IL1403, MG1363, and SK11 (4,

29, 50). Besides the genes for these enzymes, strain-specific prophage-encoded lytic activities are present (12, 49).

The PG hydrolases of *L. lactis* are modular enzymes that are composed of a signal sequence, an active-site domain, and a (putative) cell wall binding domain (21, 45). AcmA is the best-studied enzyme: the mature form comprises an N-terminal *N*-acetyl glucosaminidase active site (43) and a C-terminal domain that consists of three lysin motif (LysM) modules (8). The enzyme binds to PG in the lactococcal cell wall in a noncovalent manner via its LysM-containing domain (42, 44). Binding of AcmA has also been shown to occur intercellularly; that is, AcmA produced by and liberated from one cell can bind to and lyse another cell (7).

To control the function of these potentially suicidal enzymes, both the expression and activity of cell wall hydrolases are well regulated. Expression of the genes is low and growth phase dependent (7, 21, 45). The different enzymes have different pH dependencies: AcmD and YjgB are active at a pH of around 4, AcnC is active below pH 7, and AcmA has been shown to be active below pH 10 (21, 45). AcmA is subject to proteolytic degradation by the extracellular lactococcal proteases PrtP and HtrA, resulting in reduced activity and/or reduced wall binding (6, 8, 34, 42, 43). Recently, it has been shown that modification of the PG may also be a manner to control the activity of AcmA. Veiga et al. (48) showed that increased O-acetylation of the PG of *L. lactis* results in resistance against AcmA, possibly by reduced binding of AcmA. De-N-acetylation of lactococcal PG did not affect the binding of AcmA (31). Secondary cell wall polymers such as S-layer proteins and lipoteichoic acids (LTAs) regulate the activity of AcmA by hindering its binding to PG (44). Although PG is present all over the lactococcal cell surface, AcmA binds only at specific sites on the lactococcal cell, predominantly around the septum and poles (44). When cells are boiled in trichloroacetic acid (TCA) and AcmA is subsequently added from the

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Reference or source
<b>Strains</b>		
<i>L. lactis</i> strains		
MG1363	<i>L. lactis</i> subsp. <i>cremoris</i> ; Lac <sup>-</sup> PrtP <sup>-</sup> ; plasmid-free derivative of NCDO712	16
MG1363 <i>acmAΔI</i>	Derivative of MG1363 carrying a 701-bp SacI/SpeI deletion in <i>acmA</i>	8
SK110	<i>L. lactis</i> subsp. <i>cremoris</i> ; phage resistant; contains galactosyl in LTA	41
IL1403	<i>L. lactis</i> subsp. <i>lactis</i> ; plasmid-free strain	13
IL1403 <i>acmA::ISSI</i>	<i>acmA</i> mutant of IL1403	This study
NZ9000 <i>acmAΔI</i>	Derivative of NZ9000 carrying a 701-bp SacI/SpeI deletion in <i>acmA</i>	45
NZ9700	Nisin-producing transconjugant of strain NZ9000 containing the nisin-sucrose transposon Tn5276	23
<i>E. coli</i> EC101	<i>E. coli</i> JM101 with <i>repA</i> from pWV01 integrated in chromosome; Km <sup>r</sup>	26
<b>Plasmids</b>		
pGh9::ISSI	Used in insertional mutagenesis; Em <sup>r</sup>	28
pNG3041	Cm <sup>r</sup> ; pNZ8048 derivative containing a fusion of the pre-pro sequence of <i>prtP</i> to <i>msa2</i> and the C-terminal domain of <i>acmA</i> under control of P <sub>nisA</sub>	44

outside, AcmA binding occurs at the whole cell surface. A component that can be extracted from the cell wall by the TCA treatment, possibly LTA, is involved in hindering AcmA binding such that AcmA binds only to the mentioned sites (44). Indeed, for *L. lactis* strain SK110, it was shown that its LTAs are present at the sites in the cell wall where AcmA does not bind (44). LTAs are lipid-linked carbohydrates consisting of polyglycerolphosphate in *L. lactis*. They are involved in the control of autolysin activity (3, 15), in determining the electrochemical properties of the cell wall (33), in establishing a magnesium ion concentration (1, 19, 22, 25), and in determining the physicochemical properties of the cytoplasmic membrane (18). In the mid-1970s it was already shown that the teichoic acid moiety of the LTA of *L. lactis* contains 16 to 17 glycerolphosphate units, of which around 50% are replaced with  $\alpha$ -D-galactosyl residues (51). The glycerolphosphate was shown also to be replaced with D-alanine. Differences in the amounts of D-Ala have been reported to influence autolysis, possibly by affecting the degradation of the autolysin AcmA by the extracellular protease HtrA (42, 43).

Riepe et al. (38) observed that when the highly autolytic *L. lactis* subsp. *cremoris* strains CO and 2250 were grown in a medium containing lactose or galactose as the sole carbon source, the reduction of the optical density at 600 nm (OD<sub>600</sub>) during the stationary phase was less than when the strains were grown in a medium containing glucose. However, a molecular mechanism for this phenomenon was not described. In this paper the same phenomenon is described for *L. lactis* subsp. *cremoris* MG1363 and *L. lactis* subsp. *lactis* IL1403. We show that AcmA binding to cells grown in a medium containing galactose as the carbon source is reduced, which explains the reduced cellular autolysis observed under these specific growth conditions. The composition of the LTAs of cells grown on galactose and glucose differs considerably, which could explain the differences in autolysin binding.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, growth media, growth conditions, and chemicals.** Plasmids and bacterial strains used in this study are listed in Table 1. *L. lactis* was grown as standing cultures at 30°C or 37°C in M17 broth (Difco Laboratories, Detroit, MI) containing (in wt/vol for all sugars) the following: 0.5% glucose (GM17), 0.5% galactose, or a mixture of 0.25% glucose and 0.25% galactose.

GM17 agar plates contained 1.5% (wt/vol) agar. For the selection of plasmid pNG3041 in *L. lactis* NZ9000 *acmAΔI*, chloramphenicol (Sigma-Aldrich, St. Louis, MO) was added (5  $\mu$ g/ml). Erythromycin (Boehringer GmbH, Mannheim, Germany) was added to a concentration of 5  $\mu$ g/ml when *L. lactis* IL1403 (harboring the vector pGh9::ISSI) was grown at 30°C and to a concentration of 2  $\mu$ g/ml when incubation was at 37°C. *Escherichia coli* was grown in tryptone yeast extract medium (Difco Laboratories) at 37°C with vigorous agitation or on tryptone yeast extract medium solidified with 1.5% (wt/vol) agar and containing 100  $\mu$ g of erythromycin (Boehringer GmbH) per ml when required. For *E. coli* EC101, 40  $\mu$ g/ml kanamycin (Boehringer GmbH) was used (26). All chemicals used were of analytical grade and, unless indicated otherwise, obtained from Merck (Darmstadt, Germany).

**General DNA techniques and transformation.** Molecular cloning techniques were performed essentially as described by Sambrook et al. (40). Restriction enzymes and T4 DNA ligase were obtained from Boehringer GmbH and were used according to the instructions of the supplier. Genomic DNA of *L. lactis* was isolated as described by Buist et al. (8). Plasmid DNA was isolated at large scale using a Nucleobond Kit PC 100 (Machery-Nagel, Düren, Germany) as specified by the supplier. *E. coli* and *L. lactis* were transformed by electroporation using a Gene Pulser (Bio-Rad Laboratories, Richmond, CA) as described by Zabarovskiy and Winberg (52) and Leenhouts and Venema (27), respectively.

**Screening for transposon mutants and generation of plasmid-free derivatives.** Mutagenesis of *L. lactis* IL1403 with pGh9::ISSI and isolation of stable ISSI insertion mutants by excision of integrated vector were performed as described previously (28). Cell wall-hydrolyzing activity was visualized as a halo around colonies grown for 36 to 48 h at 37°C on GM17 plates containing 0.2% (wt/vol) autoclaved, lyophilized *Micrococcus lysodeikticus* ATCC 4698 cells (Sigma Chemical Co., St. Louis, MO) (26). Plasmid-free derivatives of the pGh9::ISSI insertion mutants of *L. lactis* IL1403 lacking a halo were generated from an overnight culture of the mutant diluted 10<sup>6</sup>-fold in fresh GM17 (without erythromycin) medium and grown for 18 h at 28°C. After growth the culture was diluted and plated onto GM17 plates to obtain single colonies. The removal of pGh9::ISSI was verified by the absence of growth of the mutant on GM17 plates containing erythromycin.

**Southern transfer, DNA hybridization, and nucleotide sequencing.** To verify single-site integration of pGh9::ISSI and to determine the restriction sites flanking the plasmid, chromosomal DNA of selected integrants of *L. lactis* IL1403 *acmA::pGh9::ISSI* was cut with the restriction enzymes ClaI, EcoRI, HindIII, or PstI. Separation was performed by electrophoresis in a 0.8% (wt/vol) agarose gel, and DNA was transferred to GeneScreen Plus membranes (NEN Research Products, Boston, MA) by the protocol of Southern analysis, as modified by Chomczynski and Qasba (11). Labeling of pGh9::ISSI digested with HindIII and hybridization (at 42°C) were done with an ECL labeling and detection system according to the instructions of the manufacturer (Amersham International, Amersham, United Kingdom). The site of integration of pGh9::ISSI was determined by rescue of the plasmid from the chromosome through digestion of chromosomal DNA of the integrants with HindIII, circularization, and introduction into *E. coli* EC101, with selection for erythromycin resistance. The inserts of rescued plasmids were sequenced using a Vistra Systems 725 automated fluorescent DNA sequencer (Vistra Systems Amersham Life Science Inc., Bucking-

hamshire, United Kingdom). Sample preparation and sequence reactions were performed with the DNA sequencing robot, the Vistra Systems DNA labstation 625, using an automated  $\Delta$ Taq sequencing kit and the Texas-Red M13 forward primer of Amersham, according to the instructions of the supplier.

**OD measurements, nisin induction, enzyme assays, and microscopy.** ODs of cultures were measured at 600 nm in a Novaspec II spectrophotometer (Pharmacia Biotech AB, Uppsala, Sweden). To produce MSA2cA protein, *L. lactis* NZ9000 (24) containing plasmid pNG3041 was grown in GM17 broth containing chloramphenicol at 30°C. The culture was grown until an OD<sub>600</sub> of 0.5 was reached, after which it was induced with nisin by the addition of 1/1,000 (vol/vol) of a supernatant of a culture of the nisin-producing strain *L. lactis* NZ9700.

To measure the influence of the carbon source on cellular lysis, *L. lactis* MG1363, *L. lactis* MG1363 *acmAΔ1*, *L. lactis* IL1403, and *L. lactis* IL1403 *acmA::ISS1* were grown in GM17 medium containing glucose (0.5%, wt/vol), galactose (0.5%, wt/vol), or a mixture of both sugars (0.25% [wt/vol] of each). Cells from 50 ml of culture of *L. lactis* MG1363 *acmAΔ1* or *L. lactis* IL1403 *acmA::ISS1* were subsequently resuspended in 50 ml of the supernatants of the *L. lactis* MG1363 or *L. lactis* IL1403 cultures and incubated at 30°C for 96 h. Subsequently, as a measure of the extent of culture lysis, X-prolyl dipeptidyl aminopeptidase (PepX) was measured using the chromogenic substrate Ala-Pro-p-nitroanilid (Bachem Feinchemicalien AG, Bubendorf, Switzerland) as described earlier (6). Briefly, after 2 min of centrifugation in an Eppendorf microcentrifuge, 75 μl of a culture supernatant was added to 50 μl of substrate (2 mM) and 75 μl of HEPES buffer (pH 7.0). The mixture was pipetted into a microtiter plate well, and color development was monitored in a THERMOMax microtiter plate reader (Molecular Devices Corporation, Menlo Oaks, CA) at 405 nm for 20 min at 37°C.

Light microscopy pictures of *L. lactis* MG1363 *acmAΔ1* and *L. lactis* IL1403 *acmA::ISS1* grown in M17 containing glucose or galactose were made by using a Zeiss microscope (Carl Zeiss, Thornwood, CA) and an Axiovision digital camera (Axion Technologies, Houston, TX). To determine the effect of AcmA on bacterial chain length, *L. lactis* MG1363 *acmAΔ1* and *L. lactis* IL1403 *acmA::ISS1* cells (pellet of 1 ml of culture) were incubated with *L. lactis* MG1363 or *L. lactis* IL1403 supernatants (supernatant of 1 ml of culture) for 30 min at 30° before microscopy. Electron microscopy was performed as described earlier (10).

**Isolation and TCA treatment of cell walls and MSA2cA binding assay.** Walls of cells grown in M17 medium with glucose or galactose were isolated and treated with TCA as described before (44). MSA2cA binding studies were performed by mixing equal amounts of *L. lactis* cells (the amount of cells present in 1 ml of culture with an OD<sub>600</sub> of 1.0) or cell walls (1 μg) with 1 ml of supernatant of a nisin-induced *L. lactis* NZ9000 *acmA* (pNG3041) culture, which contains the fusion protein MSA2cA (44). The suspensions were incubated at room temperature for 5 min, centrifuged (cells for 1 min at 20,000 × *g* and cell walls for 15 min at 20,000 × *g*) and washed once with M17 broth. The pellets were subsequently resuspended in sodium dodecyl sulfate (SDS) sample buffer (2), boiled for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (see below).

**SDS-PAGE, AcmA zymograms, and Western hybridization.** AcmA activity was detected by a zymogram staining technique using SDS-polyacrylamide (12.5%) gels containing 0.15% autoclaved, lyophilized *M. lysodeikticus* ATCC 4698 cells (Sigma-Aldrich), as described previously (8). After electrophoresis, the gels were gently shaken at room temperature for 24 h in three to five changes of 100 ml of 25 mM Tris-HCl (pH 7) containing 1% (vol/vol) Triton X-100 for protein renaturation. Bands of lytic activity became visible as clearing zones. A prestained broad-range SDS-PAGE molecular weight marker of Bio-Rad laboratories was used as a reference. Proteins were transferred from SDS-10% polyacrylamide gels to polyvinylidene difluoride membranes (Roche Molecular Biologicals, Basel, Switzerland) as described by Towbin et al. (46) for Western hybridizations. TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) was used as a buffer, and 5% skim milk powder in TBST was used to block membranes, usually overnight. Antibodies were added to TBST, and incubations were performed at room temperature for 1 h.

MSA2cA antigen was detected with a rabbit polyclonal anti-MSA2 antiserum diluted 10,000-fold and horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Pharmacia, Uppsala, Sweden) using an ECL chemiluminescent detection system and protocol (Amersham, Piscataway, NJ).

**LTA isolation and analyses.** LTA was isolated as follows: *L. lactis* IL1403 was grown overnight in 5 liters of M17 medium supplemented with 0.5% glucose or 0.5% galactose. Twenty grams (wet weight) of cells was harvested by centrifugation, resuspended in 60 ml of 0.1 M sodium citrate buffer, pH 4.8, and disrupted in three cycles of 5 min at output level 6 using a Branson sonifier (model 250 P5; Branson Corp., Danbury, CT). Between cycles the cells were kept on ice for 5 min. LTA purification was done essentially as described by Morath et al.

(32). Briefly, the disrupted cells were mixed with 60 ml of *n*-butanol (Merck, Darmstadt, Germany), stirred for 30 min at room temperature, and centrifuged at 13,000 × *g* for 40 min at room temperature. The centrifugation step was lengthened to obtain a higher yield of LTA. The aquatic phase was lyophilized, filtered using a 0.22-μm-pore-size nitrocellulose membrane filter (Schleicher & Schuell, 's-Hertogenbosch, The Netherlands), resuspended in 5 ml of chromatography start buffer (15% *n*-propanol in 0.1 M ammonium acetate, pH 4.7), and centrifuged at 45,000 × *g* for 15 min (Sorvall rotor SS34; Newtown, CT) at room temperature. The supernatant was subjected to fast-performance liquid chromatography on octyl-Sepharose CL 4B (Amersham Pharmacia Biotech), with a 15 to 60% propanol gradient.

The phosphorous content of LTA was estimated according to the protocol of Rouser et al. (39). Nuclear magnetic resonance analysis was performed at 600.13 MHz (<sup>1</sup>H) and 300 K, as described by Morath et al. (32).

## RESULTS

**Selection and isolation of an AcmA mutant of *L. lactis* subsp. *lactis* IL1403.** In previous work we have shown that AcmA of *L. lactis* MG1363 is the major lactococcal PG hydrolase (8, 43). Comparison of the genome sequences of *L. lactis* subsp. *cremoris* MG1363 and *L. lactis* subsp. *lactis* IL1403 showed that both strains possess the same chromosomally encoded PG hydrolases (50). To investigate and compare the effect of carbon sources on the activity of the major autolysin in both subspecies, we constructed an *acmA* mutant of *L. lactis* IL1403. As selection for an *acmA* deletion mutant on plates containing *M. lysodeikticus* cell wall fragments was previously shown to work well (26), this screening method was used to isolate an *acmA* mutant of *L. lactis* IL1403. A transposon mutant library of *L. lactis* IL1403 was generated using plasmid pGh9::ISS1 and screened for loss of extracellular PG hydrolase activity as described in Materials and Methods. Two out of 3,000 colonies were found to have completely lost halo-forming ability on plates containing *M. lysodeikticus* cell wall fragments. Hybridization of different digests of the chromosomal DNA of the mutants with a probe of plasmid pGh9::ISS1 revealed that the patterns for both mutants were identical (data not shown). To determine the site of integration in one of the mutants, *L. lactis* IL1403 *acmA::pGh9::ISS1*, the regions flanking pGh9::ISS1 were isolated using plasmid rescue. After DNA isolation and plasmid sequencing, pGh9::ISS1 was found to have integrated in the *L. lactis* IL1403 *acmA* gene immediately upstream of the codon for the active-site glutamine residue, resulting in loss of production of an active protein (Fig. 1).

**AcmA of *L. lactis* subsp. *lactis* IL1403 is the strain's major autolysin and is involved in cell separation.** After excision of integrated pGh9::ISS1 from the strain, the resulting mutant *L. lactis* IL1403 *acmA::ISS1* was analyzed for the loss of AcmA activity by zymographic analysis at the natural temperature of 30°C. Hybridization of chromosomal DNA of the mutant with pGh9::ISS1 showed that the plasmid was lost and that a copy of the ISS1 was left behind in *acmA* (results not shown). As expected, no AcmA activity could be detected in cell and supernatant samples of the mutant using zymographic detection although AcmA activity was present in the samples of *L. lactis* IL1403 and *L. lactis* IL1403 *acmA::ISS1* (results not shown). Comparison of cellular lysis during the stationary growth phase showed that *L. lactis* IL1403 wild-type cells lysed slightly more than cells of *L. lactis* MG1363 (Fig. 2). While lysis of the *acmA* mutant of *L. lactis* MG1363 is completely abolished, the *L. lactis* IL1403 *acmA::ISS1* mutant still lyses to



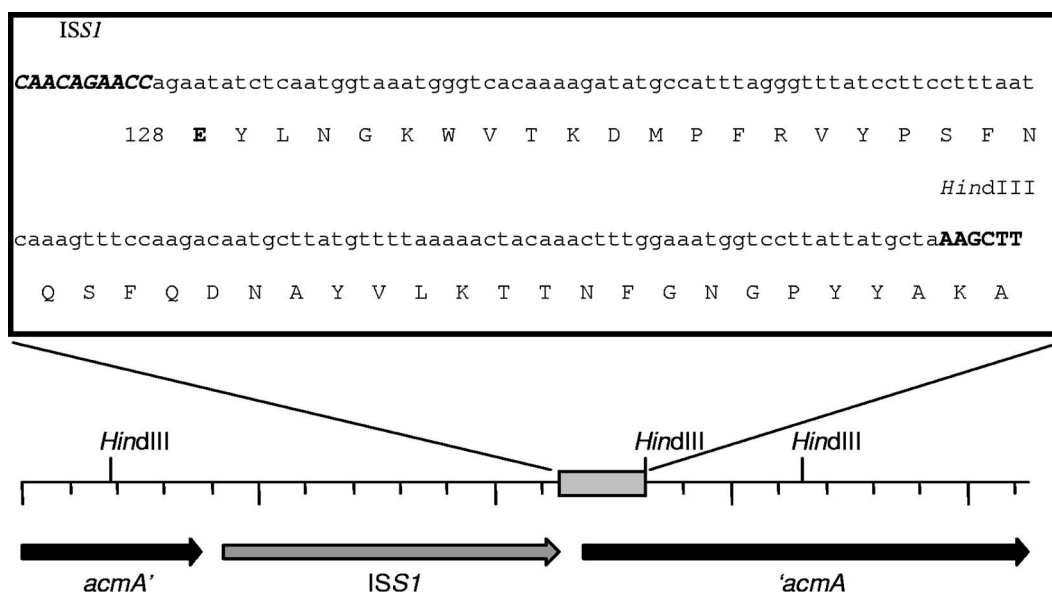


FIG. 1. Schematic presentation of the insertion of the ISS1 element in *acmA* of *L. lactis* IL1403 *acmA*::ISS1. The nucleotide sequence on the right-hand side of the inserted ISS1 element (italicized nucleotides), determined after plasmid rescue using a HindIII digestion (see Materials and Methods), is indicated in the inset. This region is indicated as a gray box in the scheme. The amino acid sequence of part of AcmA is indicated below the nucleotide sequence.

some extent. As the numbers of bacterial genes encoding PG hydrolases are identical in both strains, this difference in lysis may be due to the difference in prophage-encoded lysis modules. The relative reduction of lysis of both the *L. lactis* IL1403 and *L. lactis* MG1363 *acmA* mutants to their respective parental strains is comparable.

After overnight growth in liquid medium, the sedimentation of cells and chain elongation of *L. lactis* IL1403 *acmA*::ISS1 were observed, as previously described for the *acmA* mutant of *L. lactis* MG1363 (8), indicating that the PG hydrolase AcmA of *L. lactis* IL1403 is also involved in autolysis and cell separation.

**Growth of *L. lactis* on galactose results in lower stationary-phase lysis than when cells are grown on glucose.** Riepe et al. (38) observed that when *L. lactis* subsp. *cremoris* strains CO and 2250 were grown on a medium containing galactose as the carbon source, these strains lysed to a lesser extent than when grown on a medium containing glucose, suggesting that the carbon source influences the autolysin activity. To further investigate the effect of galactose on autolysis, *L. lactis* strains MG1363 and IL1403 were grown in M17 medium supplemented with 0.5% glucose, 0.5% galactose, or a mixture of both sugars (0.25% of each sugar) for 48 h. The release of the intracellular enzyme PepX in the culture supernatant was taken as a marker for cellular lysis and was followed in time. Lower PepX activities were measured after 25 and 48 h in supernatants of cultures grown on galactose than in cultures grown on glucose (Fig. 3). When both sugars were added in equal amounts to the growth medium, intermediate levels of PepX activity were observed. PepX expression is not influenced by the carbon source since cell extracts of cells grown on glucose or galactose contained equal amounts of PepX activity (results not shown).

Similar amounts of AcmA were present in supernatants of cultures grown on glucose, galactose, or on the sugar combination, as was attested with a zymogram assay: no differences

in clearing zones, resulting from AcmA activity, were observed between samples taken from cultures grown on glucose, galactose, or on the mixture of both sugars (results not shown). AcmA expression, therefore, is not influenced by the carbon source, and differences therein are thus not the cause of the observed reduced lysis of galactose-grown lactococcal cells.

**Lactococcal cells grown on galactose are less susceptible to lysis by AcmA.** To further examine whether growth on glucose or galactose influences AcmA-mediated cell lysis, *L. lactis* MG1363 *acmA*Δ1 and *L. lactis* IL1403 *acmA*::ISS1 were grown in the presence of glucose, after which the cells were collected. The cell pellets were resuspended in spent supernatants containing AcmA from *L. lactis* MG1363 cultures grown on glucose, galactose, or the sugar mixture. By using this approach, the influence of degradation of AcmA in the spent supernatant by proteinases that may be secreted from the mutant cells or of lysins possibly expressed from prophages in these cells (49) was excluded as the mutant cells do not lyse (*L. lactis* MG1363 *acmA*Δ1) or lyse only very slightly (*L. lactis* IL1403 *acmA*::ISS1) (Fig. 2). Cellular lysis was determined by measuring the OD<sub>600</sub> decrease of the cell suspensions and by the PepX activity in the supernatants after 96 h of incubation at 30°C. Similar amounts of PepX were released in each case, independent of the supernatant used, confirming the zymographic data that similar amounts of AcmA are present in the supernatants of *L. lactis* MG1363 grown on glucose and/or galactose (Table 2). Subsequently, *L. lactis* MG1363 *acmA*Δ1 and *L. lactis* IL1403 *acmA*::ISS1 were grown on glucose, galactose, or a mixture of both sugars until stationary phase. Equal amounts of cells were collected and mixed with the supernatant of overnight cultures of *L. lactis* MG1363 grown on glucose, galactose, or the sugar mixture. *L. lactis* MG1363 *acmA*Δ1 and *L. lactis* IL1403 *acmA*::ISS1 grown on galactose released less PepX into the supernatant after incubation with AcmA than the strains grown on glucose, independent of the source of AcmA

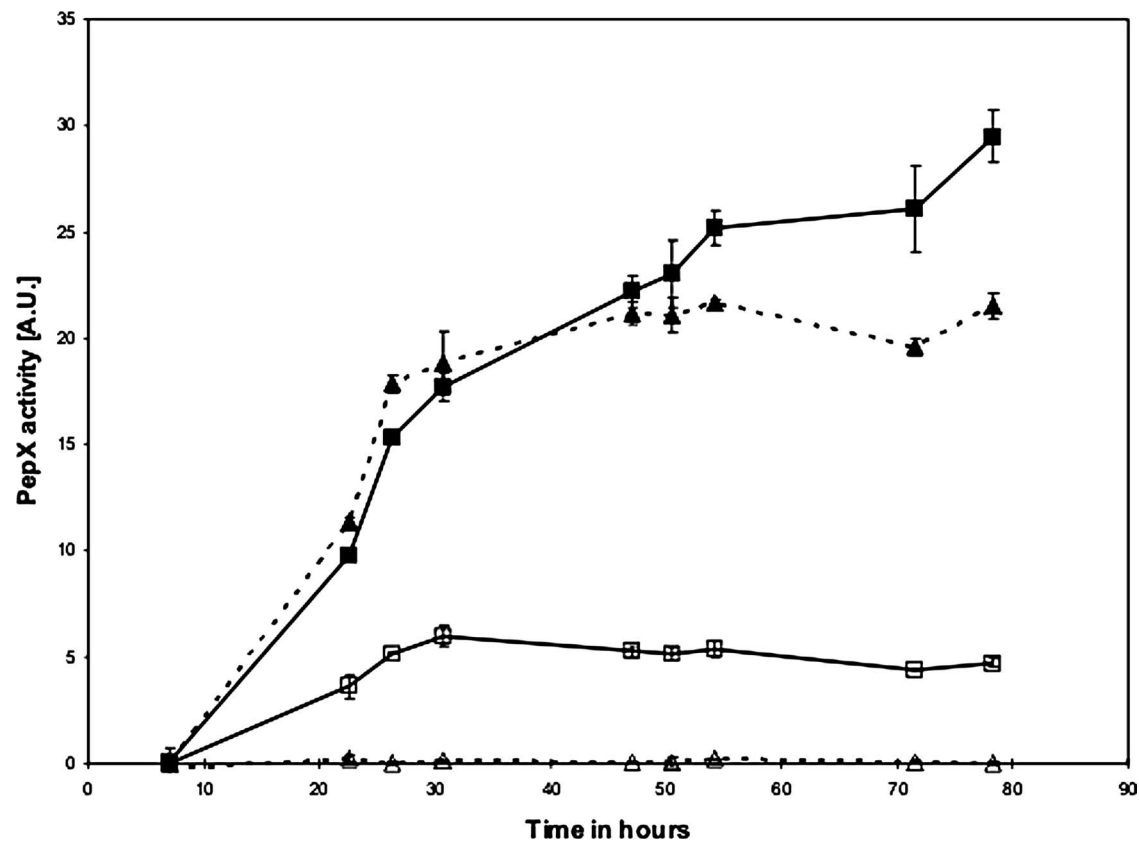


FIG. 2. Comparison of autolysis of *L. lactis* strains MG1363 (▲), MG1363 *acmAΔ1* (△), IL1403 (■), and IL1403 *acmA::ISS1* (□). Cells were grown in GM17 broth, and cellular lysis was subsequently followed by measuring PepX activity (in arbitrary units[AU]) (see the Materials and Methods section) released into the culture medium in time.

(Table 2; only the results of *L. lactis* MG1363 *acmAΔ1* are shown). Both mutant strains grown on the sugar mixture exhibited an intermediate extent of lysis. In conclusion, lactococcal cells grown on galactose are less susceptible to lysis by AcmA than lactococcal cells grown on glucose.

**Cell separation is not affected by growth on galactose.** AcmA is involved in cell separation of *L. lactis* (8; see also above). Microscopic analysis revealed that *L. lactis* MG1363 and *L. lactis* IL1403 grown on galactose form slightly longer chains (3 to 5 cells per chain) than when grown on glucose

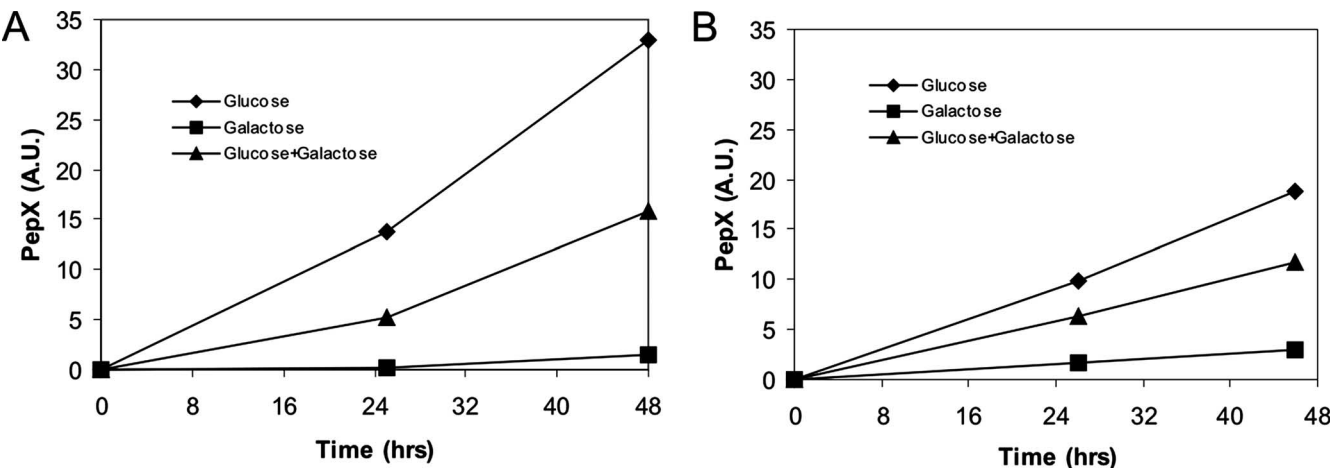


FIG. 3. Autolysis of *L. lactis* is carbon source dependent. *L. lactis* MG1363 (A) and *L. lactis* IL1403 (B) were grown in M17 medium containing (in wt/vol for each sugar) 0.5% glucose, 0.5% galactose, or a combination of 0.25% glucose plus 0.25% galactose, as indicated in the inset. Lysis was followed by measuring the PepX activity released into the culture supernatants in time. The outcome of a typical experiment is shown. Largely similar patterns were obtained in three independent experiments. AU, arbitrary units.

TABLE 2. Lysis of *L. lactis* MG1363 *acmAΔI* grown on glucose and/or galactose after incubation in *L. lactis* MG1363 spent supernatants

Carbon source in <sup>a</sup> :		% Lysis <sup>b</sup>	PepX release (AU) <sup>b</sup>
Cells (MG1363 <i>acmAΔI</i> )	Supernatant (MG1363 culture)		
Glucose	Glucose	42 (0.2)	27 (0.2)
Glucose	Galactose	42 (0.4)	30 (1.4)
Glucose	Glucose-galactose	44 (1.7)	31 (2.2)
Galactose	Glucose	28 (0.6)	21 (0.9)
Galactose	Galactose	30 (1.0)	22 (1.4)
Galactose	Glucose-galactose	29 (2.0)	23 (1.2)
Glucose-galactose	Glucose	32 (2.0)	24 (2.1)
Glucose-galactose	Galactose	35 (0.4)	25 (2.9)
Glucose-galactose	Glucose-galactose	32 (1.8)	27 (2.9)

<sup>a</sup> Sugar concentrations (wt/vol) were as follows: glucose, 0.5%; galactose, 0.5%; glucose-galactose, 0.25% of each sugar.

<sup>b</sup> The percentage of lysis was calculated using the following formula:  $100 \times \text{OD}_{600} \text{ value at 96 h} / \text{maximum OD}_{600} \text{ value}$ . All samples were taken from two independent cultures, and each sample was measured twice to determine the  $\text{OD}_{600}$  and PepX activity. Standard deviations are given in parentheses. AU, arbitrary units.

(single cells to 3 cells per chain). After prolonged incubation, however, the average chain length in all cultures is comparable (results not shown). Electron microscopy analysis revealed that cells of the *acmA* mutant of *L. lactis* MG1363 are connected via PG bridges with a density different from that of the cell wall itself (results not shown). These PG bridges seem to be hydro-

lyzed by AcmA during cell separation since separating wild-type *L. lactis* cells do not show this structure.

To study cell separation further, the two *acmA* mutant strains were grown on glucose or galactose, and when the cultures reached stationary phase, an AcmA-containing supernatant from an overnight culture of *L. lactis* MG1363 or *L. lactis* IL1403 was added to the *L. lactis* MG1363 *acmAΔI* or *L. lactis* IL1403 *acmA::ISS1* cells, respectively. The typical long chains of *L. lactis* MG1363 *acmAΔI* (8) and of *L. lactis* IL1403 *acmA::ISS1*, which are formed during growth on both glucose and galactose, were shortened up to the level of single cells after treatment with the AcmA-containing supernatant (Fig. 4). Thus, cell separation does not seem to be influenced by growth of *L. lactis* on galactose.

**Binding of AcmA to cells grown on galactose is reduced.** A deletion derivative of AcmA lacking the C-terminal PG-binding domain is not able to lyse *L. lactis* in vivo (44), suggesting that PG binding is important for activity of AcmA. To investigate whether binding of AcmA is involved in the decreased lysis of galactose-grown lactococcal cells, binding studies were performed with the C-terminal domain of AcmA. For this purpose, a fusion protein of the human malaria parasite *Plasmodium falciparum* antigen MSA2 (35) and the C-terminal PG-binding domain of AcmA of *L. lactis* MG136 was used. This protein, MSA2cA (44), was mixed with similar amounts (according to the  $\text{OD}_{600}$  of the cultures) of *L. lactis* MG1363 *acmAΔI* cells grown either on glucose or galactose. After in-

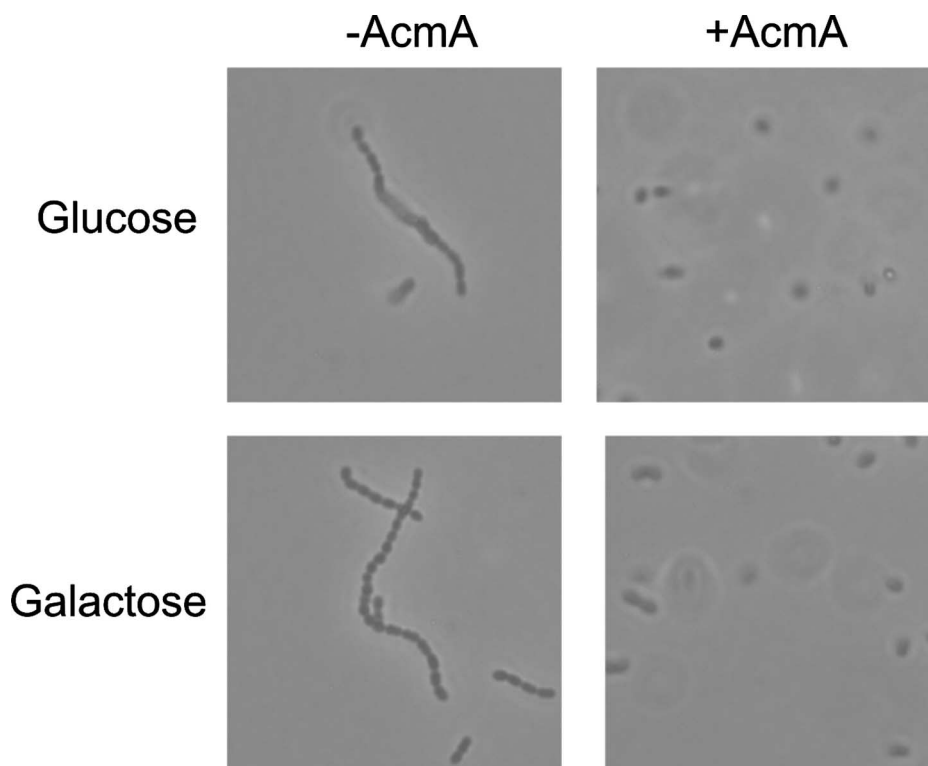


FIG. 4. Growth of *L. lactis* on galactose does not affect cell separation. Typical pictures are shown for *L. lactis* MG1363 *acmAΔI* grown on 0.5% (wt/vol) glucose or 0.5% (wt/vol) galactose. Cells were mixed with the supernatant of an overnight culture of *L. lactis* MG1363 *acmAΔI* (–AcmA) or *L. lactis* MG1363 (+AcmA). The cells were incubated for 3 h and visualized using a Zeiss light microscope and a Zeiss digital camera. Magnification,  $\times 1,000$ .

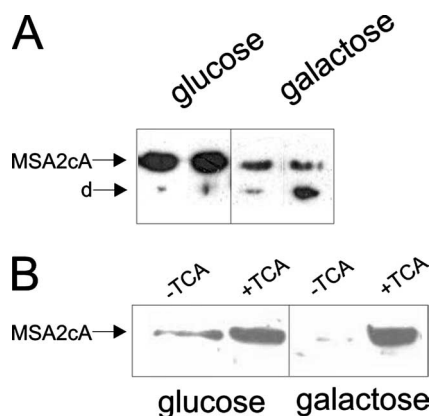


FIG. 5. Western blot showing the effects of growth of *L. lactis* on different carbon sources on the binding of AcmA via its C-terminal binding domain (cA). (A) MSA2cA binding to lactococcal cells grown on 0.5% (wt/vol) glucose or 0.5% (wt/vol) galactose. Equal amounts (as calibrated from the OD<sub>600</sub> of the cultures) of *L. lactis* MG1363 *acmAΔ1* or IL1403 *acmA::ISS1* cells grown on glucose or galactose were mixed with equal amounts of MSA2cA protein, that is, the same amount of the supernatant of an *L. lactis* culture secreting the MSA2cA protein, incubated at room temperature for 5 min, and pelleted. The pellets (cell-bound MSA2cA fraction) were analyzed by Western hybridization using anti-MSA2 antibodies. The binding experiments were performed in duplicate. d, degradation product of MSA2cA that is able to bind to the cells (still containing the cA domain). (B) MSA2cA binding to cell walls isolated from cells grown on 0.5% (wt/vol) glucose or 0.5% (wt/vol) galactose. Equal amounts of cell walls were boiled in water (–TCA) or in 10% TCA (+TCA) and used for an MSA2cA binding assay as described in panel A. Only bound fractions are shown.

cubation for 5 min at room temperature, the cells were spun down, and the MSA2cA that fractionated with the cells was analyzed by Western hybridization using anti-MSA2 antibodies (35). As shown in Fig. 5A, less MSA2cA bound to cells grown on galactose than to cells grown on glucose. The same is true for cell walls isolated from these cells: cell walls from galactose-grown cells bind less MSA2cA (Fig. 5B). However, when the cell walls were first boiled in TCA, a treatment that increases binding of MSA2cA (44), the cell types bound equal amounts of MSA2cA (Fig. 5B).

**The composition of the LTAs depends on the carbon source.** In an earlier paper we have reported that LTAs might hinder the binding of AcmA to lactococcal cell walls (42, 44). The LTAs of *L. lactis* strain SK110 were localized using fluorescent lectin and shown to be present at the sites where AcmA did not bind. The glycerolphosphates in LTA can be replaced with, e.g., alanine or galactose. To examine whether the carbon source during growth has an effect on the galactose or alanine substitutions in LTA, *L. lactis* IL1403 was grown on glucose or galactose, its LTA was isolated, and the alanine and galactose substitutions were determined (Table 3). A lower percentage of the glycerolphosphates in LTA are replaced with galactose when cells are grown on galactose ( $11.8\% \pm 1.6\%$ ) than when they grow on glucose ( $46.0\% \pm 5.9\%$  substitution rate). No significant differences in the percentages of glycerolphosphates replaced with alanines were observed between cells grown on glucose or galactose. Since only alanine and galactose substitutions in LTA were determined, it is possible that cells grown

on galactose have increased amounts of other sugars substituted in their LTAs.

## DISCUSSION

Lactococci express and secrete PG hydrolases with all kinds of functions in the cell wall and, thus, in growth and division of the cell. The lytic activity of PG hydrolases has to be controlled tightly since their activity is potentially lethal. Autolysin action is kept in check in various ways. In the case of the *L. lactis* autolysin AcmA, proteolytic cleavage by the extracellular lactococcal proteinases PrtP and HtrA results in reduced activity and/or reduced wall binding (6, 8, 34, 42, 43). Also, the C-terminal LysM-containing domain of AcmA is involved in steering enzyme activity: the autolysin binds PG in the cell wall only at places where it is needed, i.e., around the poles of the cell (44). Here, we report that the carbon source of *L. lactis* subspecies on galactose changes the carbohydrate composition or the amount of this component(s) in the cell wall such that binding of AcmA is decreased compared to growth of cells on glucose. The difference in binding of AcmA, effectuated by its cell wall-binding domain cA, is caused by a carbohydrate component on the cell wall that hinders binding but that is removable by TCA. It is not caused by a difference in the PG structure of cells grown on galactose.

The net result of these changes in AcmA binding is that cellular lysis is reduced. Decreased AcmA binding does not lead to diminished cell separation in stationary phase: cells grown on galactose still separate. Electron microscopy analysis revealed the presence of PG bridges between cells, which were present only in the *acmA* mutant of *L. lactis* MG1363 and are of a different density than the cell wall itself. The chemical composition of these PG bridges is apparently not influenced by growth on galactose, in contrast to the chemical composition of the cell wall itself.

From an earlier study it is known that LTAs are not present at the poles and septum of the lactococcal cells. As a consequence, AcmA is present only at the poles and septum of the cells (44). Growth on galactose could change the chemical composition of the lactococcal cell wall. *L. lactis* uses the Leloir pathway to metabolize galactose (17, 36). Via the same Leloir pathway, UDP-glucose and UDP-galactose are formed from galactose and/or glucose. The UDP-sugars are, besides inter-

TABLE 3. Comparison of LTAs isolated from *L. lactis* IL1403 grown on different sugars<sup>a</sup>

Carbon source	Chain length [(Gro-P) <sub>n</sub> ]	Chain substitution (%)		
		D-Ala	Galactose	Unsubstituted
Glucose	14.2 ± 8.8	14.9 ± 9.7	46.1 ± 5.9	38.6 ± 4.5
Galactose	17.1 ± 2.2	28.5 ± 6.6	11.8 ± 1.6	59.7 ± 5.2

<sup>a</sup> *L. lactis* IL1403 cells were grown on glucose (0.5%, wt/vol) or galactose (0.5%, wt/vol). LTA was isolated from *L. lactis* IL1403, purified by fast-protein liquid chromatography, and analyzed by nuclear magnetic resonance spectroscopy, as described in Materials and Methods. The nuclear magnetic resonance spectra were used to quantitate the substituents in relation to the phosphate content and to determine the average glycerolphosphate (Gro-P) chain length (*n*). Average (of two experiments) chain length and percentage of chain substitutions were determined as described in Materials and Methods and by Morath et al. (32).



mediates in the degradation of galactose to lactate, building blocks of cell wall sugars (e.g., in neutral polysaccharides and as substitutions in LTAs) (17). We presume that growth of *L. lactis* on galactose leads to differences in the amounts or ratios of UDP-glucose and UDP-galactose relative to growth on glucose. As a result, the amount or composition of the component that hinders the binding of AcmA to the PG, most likely LTA, may be changed such that less AcmA is able to bind. Since LTAs are not present at the septum and cell poles, cell separation is not influenced by growth on galactose. Interestingly, a long-chain phenotype was observed when the gene for UDP-glucose 4-epimerase (*GalE*) was interrupted in *L. lactis* MG1363 (17). Considering the above, this phenotype could be caused by reduced binding of AcmA to the *galE* mutant cells, which may contain LTAs with less galactose.

AcmA is a LysM domain-containing protein (8, 9). The LysM domain, in the C terminus of the protein (cA), constitutes a PG binding domain that has been used in a biotechnological application, namely, to bind antigens to certain gram-positive bacteria or cell walls for oral immunization purposes (5, 47). Hybrid proteins composed of this LysM-containing domain cA and an antigenic domain are produced and secreted from *L. lactis*. After isolation of the fusion proteins from the supernatant, they are loaded onto non-genetically modified gram-positive bacteria. The results from this study indicate that growth of the *L. lactis* production strain in medium containing galactose could enhance the yield of secreted fusion proteins.

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